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Pre-ESCRT Step of Endocytosis Pathway Regulates Parkinson's Disease Protein alpha-Synuclein

Jaime Pérez Pineda
Lake Forest College

Michael Fiske
Lake Forest College

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Jaime Pérez and Michael Fiske*
Department of Biology
Lake Forest College
Lake Forest, Illinois 60045

Abstract

Parkinson's disease (PD) is an incurable neurodegenerative disorder linked to the accumulation, misfolding, and aggregation of the α-synuclein protein in midbrain dopaminergic neurons. Enhancing α-synuclein degradation may be an effective goal for future drugs. Strong evidence links the proteasome and lysosome as degradation sites for α-synuclein. Recently, α-synuclein was found associated with plasma membranes and outside of cells, suggesting that α-synuclein might use the MVB/endocytosis pathway as a route to the lysosome. We tested this hypothesis in a *S. cerevisiae* model for PD. In yeast gene deletion strains lacking *vps34* or *vps27* (both involved in the initial pre-ESCRT step in this pathway), we assessed if α-synuclein accumulated, changed cellular localization, and induced toxicity. We report three significant findings. Firstly, α-synuclein altered its cellular localization in both deletion strains. Secondly, α-synuclein accumulated in *vps34*Δ cells. Lastly, the absence of either gene had no effect on toxicity. Together, our results suggest that the pre-ESCRT step in the MVB/endosome pathway is involved in degrading α-synuclein, although in an unexpectedly complex way.

Introduction

Parkinson's disease (PD) is an incurable neurodegenerative disease afflicting four million people worldwide. Classic symptoms include resting tremor and muscular rigidity in addition to other motor symptoms (Lozano et al. 2005). Lewy bodies, which are inclusions of misfolded and accumulated α -synuclein, are found in the substantia nigra of PD patients. Sufferers experience a loss of dopamine neurons in this area of the brain (Lozano et al. 2004; Giasson et al. 1999; Spillantini et al. 1998; Spillantini et al. 1998; Abeliovich et al. 2000). Misfolding and accumulation of wild-type (WT) α-synuclein leads to sporadic PD. Six causal genes have been identified in familial PD onset: α-synuclein (Polymeropoulos et al. 1997), *parkin* (Kitada et al. 1998), UCH-L1 (Funayama et al. 2002), DJ-1 (Bonifati et al. 2003), PINK1 (Valente et al. 2004), and LRRK2 (Paisan-Ruiz et al. 2004). α-Synuclein's flexible structure may play a role in its propensity to aggregate (Eliezer et al. 2001). The protein localizes to pre-synaptic nerve terminals (Jakes et al. 1994) and binds to phospholipid membranes (Davidson et al. 1998). Recently, it has been linked to the ER-Golgi pathway (Dixon et al. 2005). Additionally, animal models developed PD-like symptoms when WT α-synuclein or familial mutants A30P and A53T were over expressed (Masliah et al. 2000; Feany and Bender 2000; Lasko et al. 2003).

The past decade has provided tremendous insight into the PD field, but some questions on the molecular basis of PD remain unanswered. One important question that remains is whether α-synuclein accumulation is the cause of PD. A better understanding of the different pathway(s) α-synuclein is degraded by is essential for developing new treatments.

Studies have shown that proteasome inhibition leads to α -synuclein inclusion formation, highlighting it as a key component in α -synuclein degradation (Rideout and Stefanis 2002; Sawada et al. 2003; Sharma et al. 2006; Bedfort et al. 2008). Evidence for α -synuclein degradation by the lysosome through either chaperone mediated autophagy or macroautophagy is also strong (Lee et al. 2004; Cuervo et al. 2004; Vogliatzi et al. 2008; Webb et al. 2003). Therefore, α -synuclein uses autophagy, one of the two main pathways to the lysosome.

Increasing evidence also supports the role of endocytosis, the second major route to the lysosome, in α -synuclein degradation. Firstly, in yeast models with knockouts of key endocytosis genes, α -synuclein dependent toxicity is observed (Willingham et al. 2003). Secondly, WT and the other familial mutant, E46K, localize to the plasma membrane in yeasts, linking α -synuclein's degradation to endocytosis (Outeiro and Lindquist 2003). Accumulation of phosphorylated α -synuclein was found in *C. elegans* with endocytosis gene knockouts (Kuwahara et al. 2008). Thirdly, α-synuclein is found outside the cells in PD patients, and experiments showed that this extracellular accumulated α-synuclein was internalized through the endocytosis pathway and eventually degraded by the lysosome (Lee, H. J et al. 2008).

ESCRT proteins are important complexes in the endocytic pathway because they transport the substrate into the lumen of the endosome. Some ESCRT-I, -II, and -III proteins have been studied in relation to α-synuclein (Alexandra Ayala, Senior Thesis, 2009) while the rest are in the process of being studied (Jaime Perez, Senior Thesis, 2010) (Table 1). The two pre-ESCRT proteins, *vps34* and *vps27*, have not been fully analyzed, and their role in α-synuclein degradation is unknown.

My hypothesis is that α-synuclein interacts with the pre-ESCRT proteins while using the MVB/endosome pathway as a route to the lysosome. My model organism is *S. cerevisiae* (budding yeast). Yeasts are useful organisms in the study of PD (Dixon et al. 2005; Outeiro and Lindquist 2003; Willingham et al. 2003, Flower et al. 2007; Sharma et al. 2006). The MVB pathway has been conserved from yeasts to humans, making our discoveries clinically applicable (Katzman et al. 2001). In addition, the yeast genome has been sequenced and an entire gene knockout library is available for our use (Open Biosystems). In the present study, three α -synuclein PD-related properties were assessed in genetically compromised yeasts at the pre-ESCRT step: accumulation, localization, and cell toxicity. We report three significant findings. Firstly, α -synuclein altered its cellular localization in both deletion strains. Secondly, α -synuclein accumulated in cells without vps34. Lastly, no α-synuclein dependent toxicity was observed in either gene knockout.

Materials and Methods

Except for the Loss of Induction assay the following methods have all been adapted from Sharma et al. (2006) and are briefly described.

*This author wrote the paper for Biology 324: Advanced Cell Biology taught by Dr. Shubhik DebBurman.

α-Synuclein Constructs
The vectors used in this study (pYES2.1, GFP, WT α-synuclein, and the A30P and E46K familial mutants) were created as previously described in Sharma et al. (2006).

Yeast Strains
Parent strain BY4741 (mat a) and MVB knockout strains *vps27Δ*, *vps34Δ*, *vps23Δ*, *vps37Δ*, *vps22Δ*, *vps2Δ*, and *vps32Δ* were purchased from Open Biosystems.

Yeast Expression
α-Synuclein expression plasmid vectors were transformed into above budding yeast strains as described in Burke et al. (2000). Yeast cells were grown on media lacking uracil (SC-URA) for selection. Using polymerase-chain reaction (PCR), the presence of α -synuclein constructs was confirmed. The expression of α -synuclein was regulated using a galactose inducible promotor (GAL1) in the pYES2.1 vector. Yeast cells were grown overnight in SC-URA glucose (2%) media at 30ºC. Cells were washed with water and diluted to 5 x 106 cells/ml in SC-URA galactose (2%) media to induce expression and grown to the time points desired for various experiments mentioned below.

Western Analysis
Cells were grown overnight in 5 ml SC-URA glucose at 30°C, 200 rpm. They were centrifuged at 1500xg for 5 minutes at 4°C, and washed three times with 5ml water. Cells were then transferred to 25 ml of SC-URA galactose at 30°C at 200 rpm. At 24 hours, 2.5 x 107 cell density was calculated to create cell lysates. Cells were washed with 100 mM NaN3, solubilized in ESB (electrophoresis sample buffer), and small beads were added to break up contents. Samples were run on Tris-Glycine gels using SDS containing running bugger. SeeBlue was used as the molecular weight standard. Gels were transferred to a PVDF membrane. Western blot was performed by washing membranes with different antibodies using standard protocols and detected for alkaline phosphatase activity: anti-V5 to detect α-synuclein, and anti-PGK (phosphoglycerolkinase) as a loading control.

GFP Microscopy
Cells were grown overnight in 10 ml SC-URA glucose at 30°C at 200 rpm. Protein expression was induced with SC-URA galactose as previously described. After 24 and 48 hours, 1 ml of culture was pipetted into a 1.5 ml microcentrifuge tube and 10 ml cell suspension was pipetted onto a slide. Cells were visualized under a Nikon TE2000-U fluorescent microscope and images were acquired and analyzed using Metamorph 4.0 imaging software.

Growth Curve Analysis
Cells were grown overnight in 5 ml SC-URA glucose at 30°C, 200 rpm. They were centrifuged at 1500xg for 5 minutes at 4°C, and washed three times with 5 ml water. Cells were resuspended in 10 ml water and counted. 2.0 x 106 cell density was inoculated into 25 ml galactose and grown in a shaking incubator at 30°C, 200 rpm for 48 hours. At specific time points (0, 3, 6, 12, 18. 24, 36 and 48 hours), 1 ml of culture was removed from each culture and pipetted into two cuvetes for duplicate readings. Readings were taken using a Hitachi U-2000 Spectrophotometer set at 600 nm.

Spotting
Cells were grown overnight in 5 ml SC-URA glucose at 30°C, 200 rpm. They were centrifuged at 1500xg for 5 minutes at 4°C, and washed three times with 5 ml water. Cells were resuspended in 10 ml water and counted. 2.0 x 107 cell density were placed into a 1.5 ml centrifuge tube and centrifuged at 2500 rpm for 5 minutes. Water was removed and cells were resuspended in 1 ml of sterile DI water. Cells were 5-fold serially diluted into 96-microwell plates and spotted on SC-URA glucose and galactose plates using a multi-channel pipette. Photographs were taken after 24 hours of growth for glucose plates and 48 hours for galactose plates.

Loss of Induction
Cells were grown overnight in 5 ml SC-URA glucose at 30°C, 200 rpm. They were washed twice in 5 ml water. Cells were re-suspended in 10 ml water and 4 ml were transferred into 46 ml of SC-URA galactose and grown for 24 hours. At 24 hours, cells were counted and lysates were prepared. The culture was then transferred to a 50 ml centrifuge tube, washed 3 times with 10 ml water and re-suspended in 15 ml. 5 ml of culture was transferred to 45 ml of SC-URA glucose. Lysates were prepared for galactose 24 hrs, glucose 0 hrs, glucose 6 hrs, glucose 12 hrs, and glucose 24 hrs, and Western blot analysis was performed as described above.

Statistics
Western blots, loss of induction blots, and microscopy pictures at each time point were repeated twice. Growth curves and 3 sets of glucose/galactose spotting were repeated three times. Growth curves were averaged and a cumulative growth curve was generated. Two-tailed distribution, two sample equal variable t-tests were performed on all cumulative growth curves using Microsoft Excel. Each α-synuclein plasmid vector was compared to the parent plasmid. For microscopy, Metamorph 4.0 imaging software was used to count 750 DIC cells, and the corresponding fluorescent pictures of cells were scored for phenotype and percentages were calculated.

Results

Gene deletion yeast strains for individual pre-ESCRT complex proteins *vps34Δ* and *vps27Δ* were used to analyze the role of the MVB/endosome pathway in α-synuclein degradation. These strains were compared to the isogenic parent strain, BY4741. Five plasmid vectors were used. The pYES2.1 parent plasmid and GFP plasmid served as controls. The remaining vectors included WT (wild-type) α-synuclein and two α-synuclein familial mutants (A30P and E46K). The three PD-related α-synuclein proprieties examined were intracellular localization, toxicity, and accumulation. Localization was analyzed through fluorescent microscopy. Cellular toxicity was assessed through growth curves and five-fold dilution spotting, and Western blot was utilized to determine how much α-synuclein accumulated.

α-Synuclein Is Non-Toxic and Binds to Plasma Membranes in Endocytosis Intact Yeasts
The isogenic parent strain was used as a control as endocytosis is intact in this yeast strain. As previously shown in Sharma et al. (2006), α-synuclein localized to the plasma membrane in both WT and the E46K familial mutant by 48 hours, while the other familial mutant, A30P, localized at the cytoplasm at the same time points (Figure 1A). An equal amount of WT and E46K α-synuclein was expressed in the cells, while A30P was expressed at a lower level (Figure 1B). Lastly, α-synuclein was not toxic to yeasts. In fact, A30P cells grew more rapidly than the controls and other α-synuclein constructs.

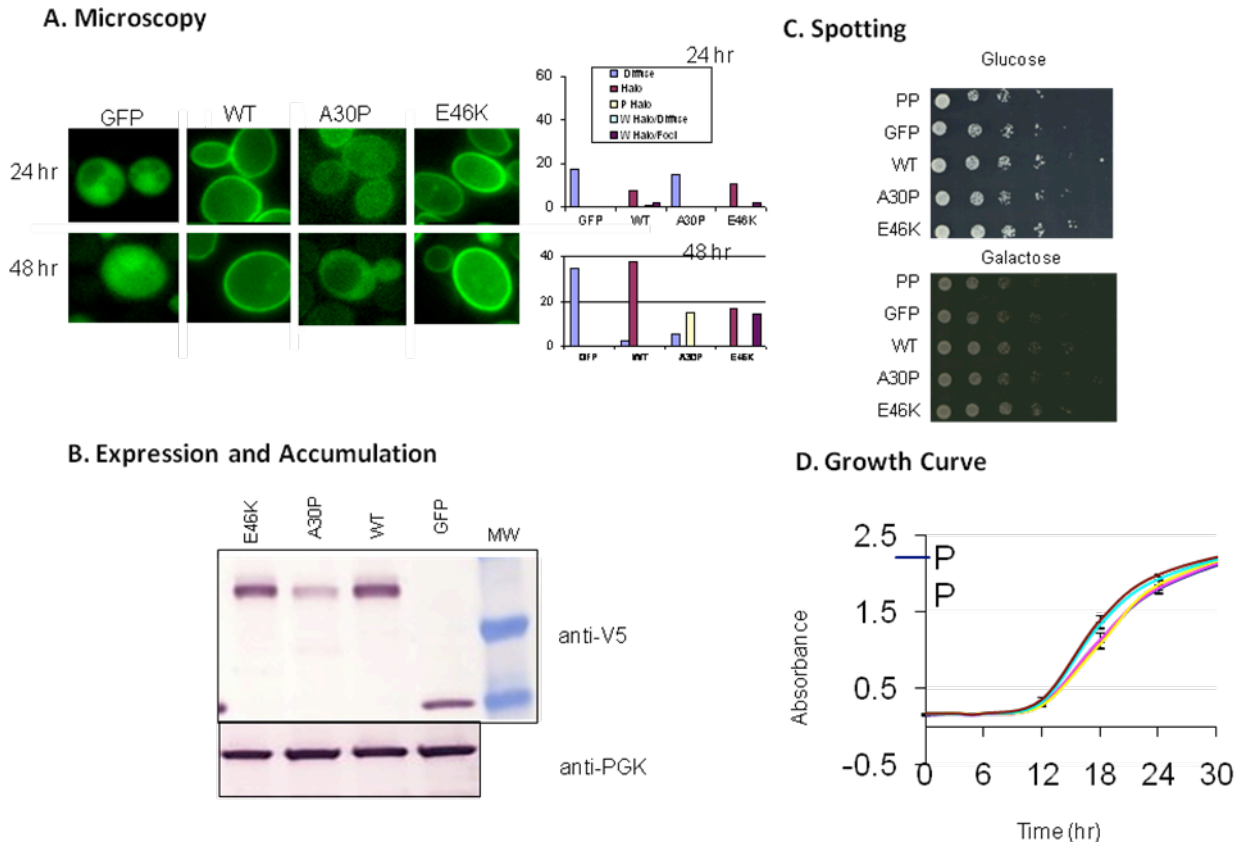

Figure 1: α-Synuclein is non toxic and binds to plasma membrane in endocytosis intact yeasts. A. Microscopy: LEFT- Live cell GFP imaging of a-synuclein distribution in BY4741 cells at 24 and 48 hrs. RIGHT- Localization Quantification of 750 cells of each transformed α-synuclein construct in BY4741, cells were counted and scored at 24 (top) and 48 hrs (bottom) post-induction for five different florescence localization patterns. B. Expression & Accumulation: Western blotting was used to assess the amount of WT and familial mutant α-synuclein present in BY4741 yeast at 24 hrs. C. Spotting: Five-fold serially diluted spotting plates for α-synuclein expressed in BY4741 in glucose (non-induced) and galactose (induced) media. D. Growth Curve Growth curve using OD600 readings for BY4741 control cells induced for WT, E46K α-synuclein and control GFP. T-tests were performed at 18 and 24 hours comparing the α-synuclein vectors to the parent plasmid at the respecting time points, all gave p-values values larger than 0.05, except for A30P at 18 and 24 hours.

α-Synuclein Accumulates in Vps34Δ Without Enhancing Toxicity

Our next goal was to examine how α-synuclein properties changed in *vps34Δ*. In support of our hypothesis, both WT and E46K α-synuclein had altered localization at 48 hours but not 24 hours. In *vps34Δ*, α-Synuclein was membrane bound and localized to the cytoplasm at 48 hours when compared to parent strain. A30P did not alter its localization pattern (Figure 2A). Moreover, WT α-synuclein showed increased accumulation in *vps34Δ* when compared to parent strain. The loss of induction assay confirmed these results. At 24 hours galactose and 0 hours glucose, *vps34Δ* expressed more WT protein than parent strain (Figure 2B). However, the expression of any form of α-synuclein did not cause toxicity to the cells when compared to parent plasmid. In fact, A30P cells again grew better (Figures 2C & D).

Increased Localization of α-Synuclein to the Cytoplasm in Vps27Δ

Finally, *vps27Δ* was analyzed to see if all pre-ESCRT proteins regulated α-synuclein similarly. Reminiscent of *vps34Δ*, WT and E46K α-synuclein localized weakly to the plasma membrane at 48 hours. In contrast, at 48 hours α-synuclein localized to the cytoplasm and at the plasma membrane. Again, A30P remained cytoplasmically localized (Figure 3A). WT, A30P, and E46K α-synuclein were expressed at similar levels compared to BY4741 (Figure 3B). Finally, none of our α-synuclein constructs induced toxicity in *vps27Δ* yeasts (Figures 3C & D).

Discussion

Understanding how α-synuclein is degraded is of significant therapeutic value. The field needs more evidence to support α-synuclein degradation through the MVB/endosome pathway to the lysosome. This paper focused on the pre-ESCRT protein components of this pathway. We report three

significant findings. Firstly, α-synuclein altered its cellular localization in both deletion strains. Secondly, α-synuclein accumulated in cells without *vps34*. Lastly, neither gene knockout enhanced α-synuclein dependent toxicity. The

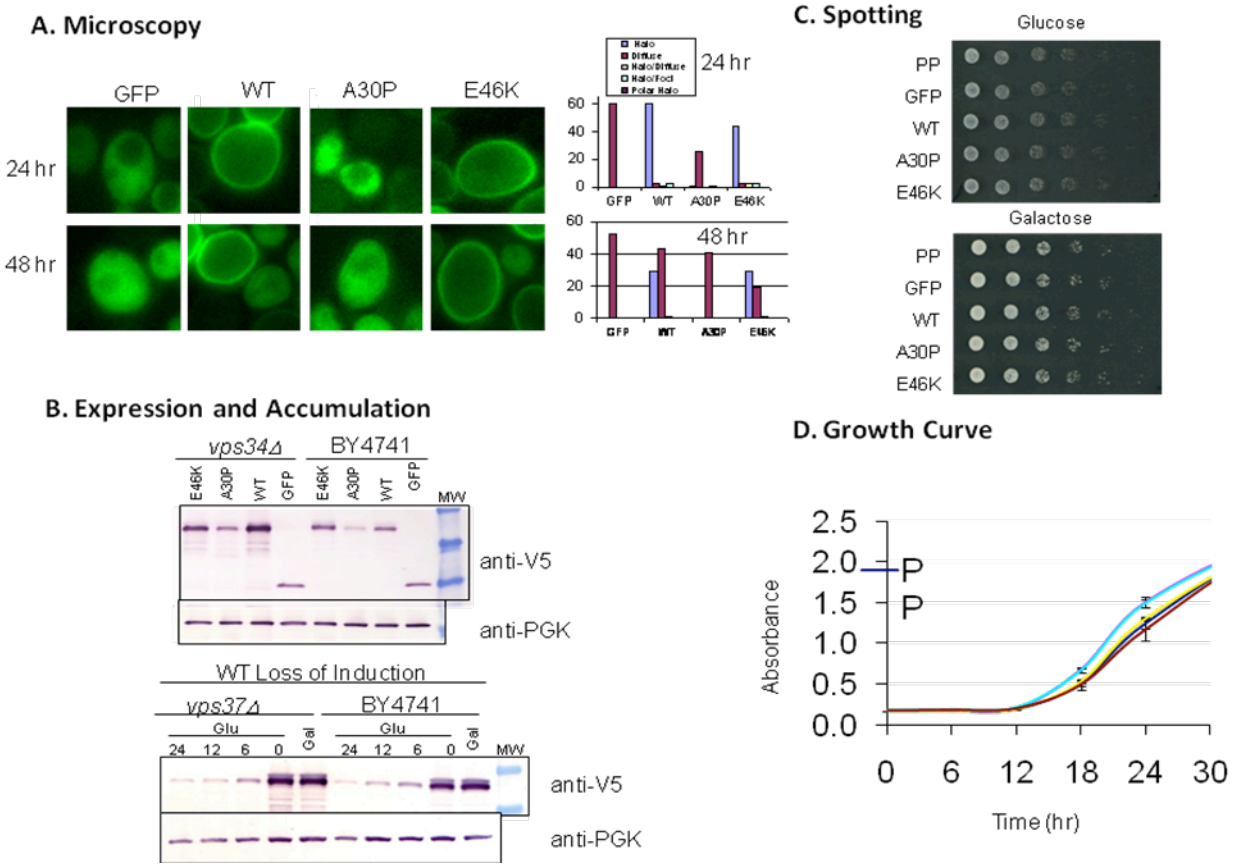

Figure 2: α-Synuclein accumulates in *vps34Δ* without enhancing toxicity. A. Microscopy: LEFT- Live cell GFP imaging of α-synuclein distribution in *vps34Δ* cells at 24 and 48 hrs. RIGHT- Localization Quantification of 750 cells of each transformed α-synuclein construct in *vps34Δ*, cells were counted and scored at 24 (top) and 48 hrs (bottom) post-induction for five different florescence localization patterns. B. Expression & Accumulation: TOP- Western blot of α-synuclein and GFP alone expression (anti-V5) at 24 hours in BY4741 and *vps34Δ* lysates. BOTTOM- Loss of Induction for WT α-synuclein in BY4741 and *vps34Δ*. PGK serves as loading control. C. Spotting: Five-fold serially diluted spotting plates for α-synuclein expressed in *vps34Δ* in glucose (non-induced) and galactose (induced) media. D. Growth Curve: Growth curve using OD600 readings for *vps34Δ* cells induced for WT, E46K α-synuclein and control GFP. T-tests were performed at 18 and 24 hours comparing the α-synuclein vectors to the parent plasmid at the respecting time points. WT and E46K gave p-values larger than 0.05. GFP gave p-values of 0.02 and 0.02 at 18 and 24 hours respectively. A30P gave p-values of 0.018 for both 18 and 24 hours when compared to parent plasmid.

DebBurman lab began evaluating this question in 2008, and our study extends this work even further.

Table 1 summarizes the progress of the work conducted on the MVB/endocytosis pathway. More research is needed to evaluate how significantly the MVB/endosome pathway's involvement is in α-synuclein degradation.

Compromised Endocytosis Regulates α-Synuclein Localization

We observed a change in localization in *vps34Δ* and *vps27Δ*, which supported our hypothesis. Studies with different yeast compromised for individual ESCRT proteins with α-synuclein have shown changes in localization as well (Table 1) (Alexandra Ayala, Senior Thesis, 2009; Jaime Perez, Senior Thesis, 2010). Previous data shows that *vps34* is required for protein and membrane trafficking events such as MVB formation (Kihara et al. 2001). *Vps27* also mediates endosomal protein sorting through ubiquitin attachment (Bilodeau et al. 2002). Since these proteins mediate such important MVB/endosome cargo components, and since α-synuclein is expected to take this MVB/endosome pathway for degradation, deleting such important genes should change α-synuclein localization.

α-Synuclein Accumulates in Vps34-Compromised Yeasts

Our second important finding is that WT α-synuclein in *vps34* accumulated, confirming our hypothesis. On the other hand, since no effect on *vps27* was noted, our hypothesis was refuted (Table 1). Since *vps34* and *vps27* have roles in MVB cargo formation, perhaps *vps27* does not play as large of a role as *vps34* in α-synuclein degradation. Previous studies demonstrated that ESCRT-II does not have a significant part in endosomal transport (Bowers et al. 2005), suggesting that not all the different ESCRT proteins are involved in the degradation of α-synuclein. *Vps37* is responsible for the synthesis of a specific phospholipid, phosphatidylinositol 3-phosphate, which then forms a complex at the membrane with *vps15* to regulate protein

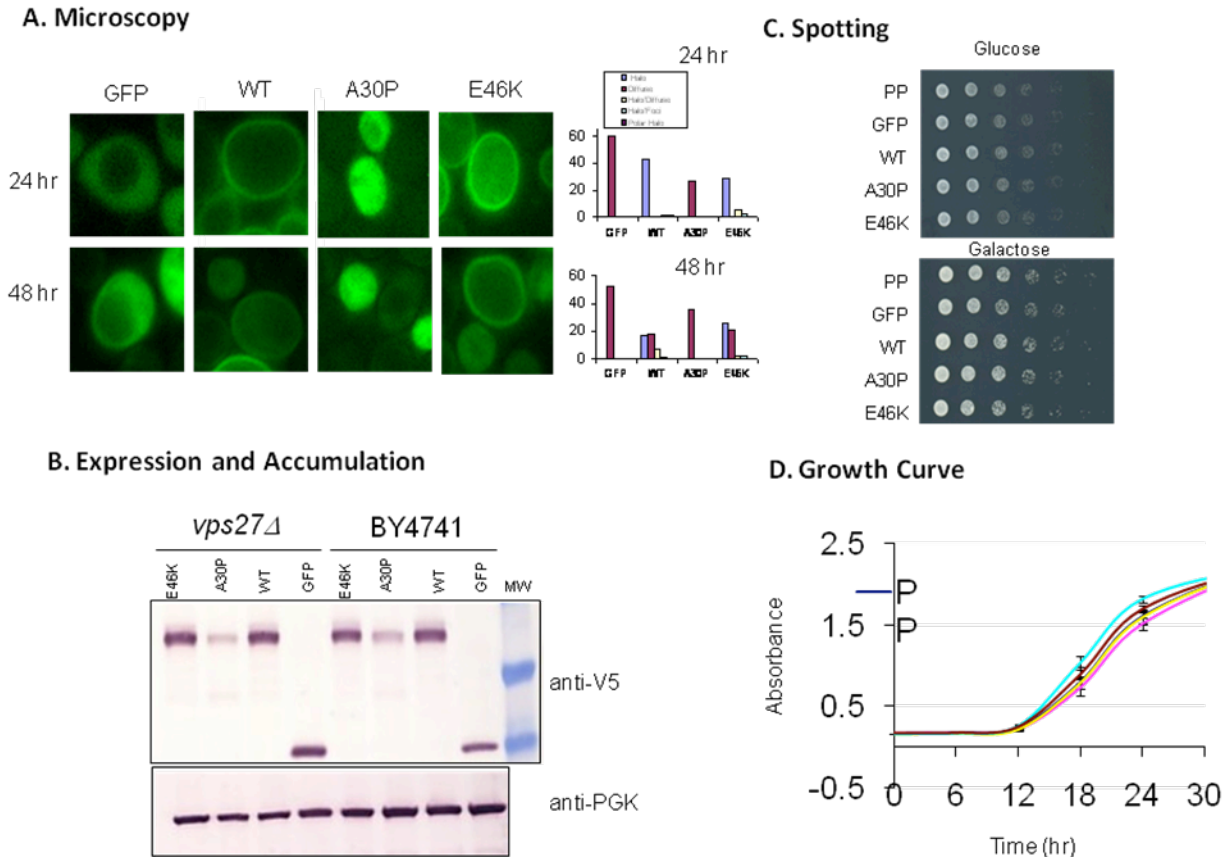

Figure 3: Increased localization of α-synuclein to the cytoplasm in *vps27Δ*. A. Microscopy: LEFT- Live cell GFP imaging of α-synuclein distribution in *vps27Δ* cells at 24 and 48 hrs. RIGHT- Localization Quantification of 750 cells of each transformed α-synuclein construct in *vps27Δ*, cells were counted and scored at 24 (top) and 48 hrs (bottom) post-induction for five different florescence localization patterns. B. Expression & Accumulation: LEFT- Western blot of α-synuclein and GFP alone expression (anti-V5) at 24 hours in BY4741 and *vps34Δ* lysates. RIGHT- Loss of Induction for WT α-synuclein in BY4741 and *vps27Δ*. PGK serves as loading control. C. Spotting: Five-fold serially diluted spotting plates for α-synuclein expressed in *vps27Δ* in glucose (non-induced) and galactose (induced) media. D. Growth Curve: Growth curve using OD600 readings for *vps27Δ* cells induced for WT, E46K α-synuclein and control GFP. T-tests were performed at 18 and 24 hours comparing the α-synuclein vectors to the parent plasmid at the respecting time points, all gave p-values values larger than 0.05.

sorting (Herman & Erm, 1990; Stack et al. 1993). *Vps27* on the other hand, forms a different complex with *Hse1*, which is a protein required for the recycling of Golgi proteins. The *vps27-Hse1* complex then aids in lumenal membrane formation and ubiquitinated protein sorting (Piper et al. 1995; Bilodeau et al. 2002). Impairment of *vps34* might have prevented creation of this important phospholipid, and subsequently, inhibited protein sorting. When this interaction was impaired, α-synuclein degradation ceased, and the protein accumulated in the cell. In addition, other proteins in the ESCRT complex could have compensated for the lack of *vps27*.

A Surprising Absence of Toxicity

Our final notable finding is the surprising absence of α-synuclein-dependent toxicity in either strain (Table 1). We expected to see cell death or impaired growth due to α-synuclein expression based on previous literature. One possibility is that the proteosome compensates when the MVB/endosome pathway is compromised and clears any toxic α-synuclein. Research demonstrates that the opposite is true; when the proteasome pathway is impaired, autophagy acts as a compensatory system (Pandey 2007). Yeasts are quite adept at surviving under harsh environmental conditions, and they might have evolved protective factors that diminish α-synuclein's toxicity. Since localization shifts to the plasma membrane, perhaps these cells need a minimum level of α-synuclein at the plasma membrane in order for toxicity to occur. Studies show that α-synuclein disrupts normal membrane processes that eventually lead to toxicity (Volles and Lansbury 2007). Lastly, samples in our lab might not be expressing α-synuclein at high enough concentrations to induce toxicity. Studies with different yeast compromised for individual ESCRT proteins with α-synuclein have also not exhibited an α-synuclein dependent toxicity (Table 1) (Alexandra Ayala, Senior Thesis, 2009; Jaime Perez, Senior Thesis, 2010).

Future Studies

Future studies need to examine the other pre-ESCRT proteins such as *Hse1*. Additionally, examining the post-

Strain:	Localization	Toxicity	Accumulation
Pre-ESCRT			
vps27Δ	**Weak**	**Non Toxic**	**Strong (WT)**
vps34Δ	**Weak**	**Non Toxic**	**None**
ESCRT-I:			
mvb12Δ	None	Non Toxic	Weak
vps23Δ	Weak	Non Toxic	Weak
vps28Δ	Weak	Weak	Weak
vps37Δ	Strong	Non Toxic	Strong
ESCRT-II:			
vps25Δ	Weak	Non Toxic	Weak
vps36Δ	Weak	Non Toxic	Strong
ESCRT-III:			
vps20Δ	Weak	Non Toxic	Strong
vps24Δ	Strong	Toxic (A30P)	None

Table 1: Deletion Analysis- Summary of Changes

ESCRT proteins and their ability to regulate α-synuclein degradation is important because of their role in the disassembly and final step of the protein sorting process. Other studies could examine the combined effect of two ESCRT protein gene deletions with α-synuclein to answer whether MVB/endocytosis genes compensate for one another. Lastly, it might be beneficial to study combined MVB dysfunction with a compromised proteasome.

Conclusion

Currently no treatment for the burden caused by neurodegenerative disorders exists. The research being done will help our understanding of the mechanism by which α-synuclein is removed from PD patients and eventually lead to the development of information that will help cure PD and similar diseases.

This study focused on one of the mechanisms by which α-synuclein is removed from cells. From the three significant findings of this study together, our results suggest that the pre-ESCRT step in the MVB/endosome pathway is involved in degrading α-synuclein, although in an unexpectedly complex way. Results support our hypothesis. While all three ESCRT complexes appear to be involved, not all of their protein components may be contributing to the regulation of α-synuclein degradation.

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